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# pH-Dependence of Extension Growth in *Avena* Coleoptiles and Its Implications for the Mechanism of Auxin Action[1]

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## ABSTRACT

**The pH-dependence of acid-induced growth in excised segments of *Avena sativa* coleoptiles has been reinvestigated in the pH range 3 to 7. In contrast to previous reports (*e.g.* DL Rayle [1973] Planta 114: 63–73), only acidic buffers with a pH below 5.0 induce an extension response. A pH of 3.5 to 4.0 is required to mimic auxin-mediated growth. Very similar pH-response curves are obtained with both intact (abraded) and peeled coleoptiles. These results agree with the recent finding of a similarly low sensitivity to protons in maize coleoptiles. It is shown that the apparently much higher sensitivity to protons previously reported for peeled *Avena* coleoptiles is due to incubating the tissue in buffer of pH 6.8 between peeling and measuring the effect of acidic buffers. Neutral pH reversibly inhibits the spontaneous extension burst originating on release from tissue tension after removing the epidermis. Reversal of this inhibition can be achieved by buffers of pH 5.0 to 6.0 (or distilled water), thereby simulating an acid-induced growth response in this pH range. It is concluded that true acid-induced wall-loosening generally does not take place above pH 5.0 and that a pH considerably below 4.0 is required in order to stimulate growth to an extent comparable to that obtained in response to auxin. The "acid-growth theory," which requires an acid-mediated loosening of the cell wall in the pH range 5 to 6, this pH being established by auxin-induced proton excretion, can therefore also not be substantiated in *Avena*.**

The "acid-growth theory" of auxin action postulated a causal role for protons in mediating the auxin-dependent cell wall loosening process leading to growth (6, 9, 20). There is general agreement that incubation of auxin-responsive tissues in acidic media of sufficiently low pH (*e.g.* pH 4) results in wall loosening and thereby in turgor-driven cell extension (3, 12, 14, 15, 23). It is equally accepted that auxin induces these tissues to excrete protons which acidify the cell-wall solution to a pH as low as about 5 (4, 5, 12–15, 23). The decisive question, however, is whether this pH is sufficiently low to account, at least initially, for the increase in growth rate caused by auxin in these cells. Using segments from *Avena* coleoptiles, Rayle (19) reported that this material is indeed very sensitive to external protons. His experiments demonstrated acid-mediated elongation of living coleoptile segments, as well as acid-mediated loosening in isolated cell walls, with an upper threshold at pH 6 and a maximum response at pH 5. In apparent agreement with the acid-growth theory, the rate of acid-mediated growth at pH 5 fully accounted for the growth rate which can be induced in *Avena* coleoptile segments with saturating concentrations of auxin. In a more recent study on the effect of acid pH on the immediate and long-term extension responses of frozen-thawed *Avena* coleoptiles stretched by an applied force, Cleland *et al.* (10) also found promotive effects in the range of pH 5 to 6 which, however, cannot be extrapolated directly to turgor-driven growth of intact tissue because of the qualitative differences between the forces involved.

In contrast to these investigations with *Avena*, a quite different pH-dependence of acid-growth has been found in recent work with maize coleoptiles (14, 16). In this material, no effect of external protons on growth and *in vitro* extensibility could be detected at or above pH 5. Moreover, matching of the auxin-mediated elongation rate with acidic buffers required an external pH of 3.0 to 3.5, well below the physiologically feasible range. Very similar pH-response curves for acid-induced elongation were obtained with intact (abraded) coleoptiles (14) and with coleoptiles from which the cuticle had been removed by peeling off the epidermis (16). This observation indicated that these experiments were not complicated by limited permeation of protons into the tissue. On the basis of this and other experimental arguments, it was concluded that protons cannot be the wall-loosening factor in auxin-mediated growth (14, 15).

The contradictory results obtained with the two species have recently led to the view (7, 8, 10) that, although protons may not represent the wall-loosening factor in auxin-mediated growth of maize coleoptiles, the acid-growth theory is still valid in the case of the auxin-mediated growth of *Avena* coleoptiles. Nevertheless, the existence of qualitatively different mechanisms of growth control by the same hormone in such closely related plants as *Avena* and *Zea* would represent a remarkable example of physiological divergence in the plant kingdom.

The aim of the present study was to resolve the discrepancies concerning the pH-dependence of the acid-mediated growth response by examining the role of some differences in methodological details occuring in the work with *Avena* (10, 19) and maize (14, 16). The effect of removing the epidermis (peeling) and preincubating the coleoptile segments in neutral buffer before inducing the acid-growth response was given particular attention. The latter treatment has previously been reported to displace the pH-response curve for acid growth in maize coleoptiles to higher pH values (14, 16).

## MATERIALS AND METHODS

Caryopses of *Avena sativa* L. cv Victory (purchased from Svalöf AB, Sweden) were sown on moist vermiculite in cov-

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ered plastic boxes and the seedlings grown for 4 d in darkness at 25.0 ± 0.3°C. A 10-min pulse of red light (0.7 W $m^{-2}$) was applied 24 h before harvesting in order to obtain straight coleoptiles of 25 to 30 mm in length. Gentle abrasion of the cuticle was achieved by pushing the coleoptiles four times through a loop of dry polishing cloth (Vitex No. 364 rouge, from Vereinigte Schmirgel- und Maschinenfabriken AG, Hannover, FRG). Coleoptiles so treated showed a regular pattern of stained patches (occupying about 30% of the epidermal area) after incubation in neutral red (5 g $L^{-1}$) for 5 min but were virtually unstained after a similar incubation in Evans blue (5 g $L^{-1}$). Since neutral red is accumulated in living cells whereas Evans blue stains only damaged cells (24), this test indicates that the abrasion procedure applied effectively disrupts the cuticle without injuring the underlying epidermal cells. Abrading coleoptiles in this way had no significant effect on subsequent growth responses and was therefore used in preference to the less gentle method previously applied with maize coleoptiles (14). Removal of the epidermis (≥90%) was carried out as described previously (16) using 15-mm segments cut 3 mm below the coleoptile tip. After being reduced to 10 mm in length, the segments were placed in a growth recorder equipped with a linear-displacement transducer which allowed the measurement of growth rate at 1-min intervals with an accuracy of ±1 $\mu$m $min^{-1}$ (14). During elongation measurements, the intact, abraded, or peeled 1-cm segments were submerged in 4 mL of distilled water, buffer, etc. at 25.0 ± 0.2°C through which a stream of air was passed continously. For preincubations, segments were strung along a stainless steel tube (0.5 mm diameter) and placed in test tubes with 20 mL of continously aerated solution (25.0 ± 0.2°C). In all experiments involving transfer from water to buffer or vice versa, the water was supplemented with 20 mmol $L^{-1}$ mannitol in order to avoid osmotic effects. Measurements with a freezing-point osmometer showed that this mannitol concentration was iso-osmotic (±3 mosmol $L^{-1}$) with all buffers used.

Data points represent means of 6-10 independent measurements ± estimates of standard error. Growth kinetics are presented as growth-recorder tracings from single representative experiments.

## RESULTS AND DISCUSSION

Typical kinetics of the auxin-induced growth response of *Avena* coleoptile segments are shown in Figure 1. In contrast to the situation in maize (14), a transient growth burst 40 min after cutting and incubating in water was consistently observed in segments from *Avena* coleoptiles (see ref. 11 for a similar observation described as a 'tactile effect'). The elongation rate reached a minimum after about 2 h, followed by a spontaneous resumption of growth at a rate of 2.5 ± 1 $\mu$m $min^{-1}$. Addition of IAA after 2 h of preincubation increased the growth rate to 8 ± 1 $\mu$m $min^{-1}$ after a lag of 12 ± 2 min. Gentle abrasion of the cuticle with a polishing cloth had no significant effect on the growth kinetics.

Figure 2 shows the effect of acid buffers on the elongation of abraded segments. In osmotically controlled experiments, 7 mmol $L^{-1}$ citrate buffer induces a rapid increase in the elongation rate at pHs below 5 whereas, compared to the water control, elongation is inhibited in the pH range

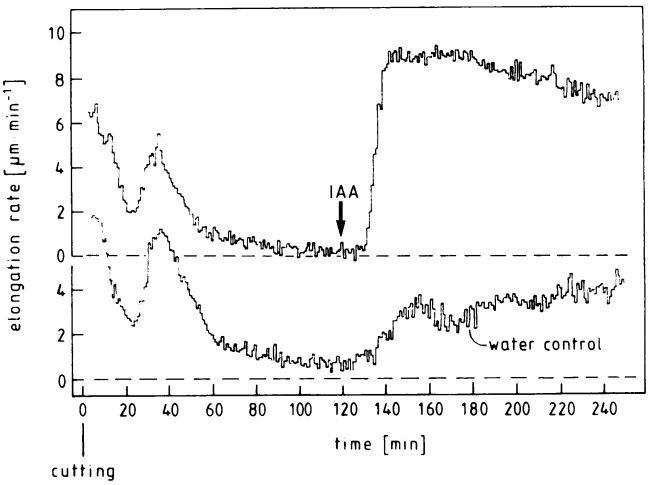


**Figure 1.** Kinetics of IAA-induced elongation in *Avena* coleoptile segments. Nonabraded segments were introduced into the growth recorder immediately after cutting and incubated in distilled water. IAA (10 $\mu$mol $L^{-1}$) was added 2 h after cutting (arrow).

above 5. Basal elongation in water corresponds to elongation in buffer of about pH 5, indicating that the effective apoplastic pH in the untreated segments is close to pH 5. Stabilization of a pH around 5 (2), rather than a more neutral pH (4), is to be expected on account of the production of respiratory $CO_2$ in this metabolically very active tissue. In order to induce a (transient) increase in elongation rate equal to the steady state rate obtained with IAA (8 $\mu$m $min^{-1}$) the acidity of the medium must be lowered to pH 3.5 to 4.0. The rapidity of the response to external pH shows that abrasion effectively removes the cuticular permeation barrier for protons. Very similar results were obtained with buffers containing only 1 mmol $L^{-1}$ citrate (data not shown). These data closely resemble the pH-dependence of acid growth found in maize coleoptiles (14) but deviate significantly from the related data for *Avena* previously reported by Rayle (19).

The discrepancy between the results of Rayle (19) and those shown in Figure 2 could be due to the fact that Rayle used coleoptile segments from which the epidermis had largely been removed by peeling. The possibility exists, therefore, that the epidermis restricts the action of acidic buffers either by mechanically inhibiting extension or by limiting the entrance of protons into the inner coleoptile tissues. This possibility is investigated in Figure 3. Nongrowing segments from which at least 90% of the epidermis has been peeled off directly before the growth measurement expand rapidly by water uptake due to the release from the longitudinal tissue tension which had previously been imposed on the inner cells by the epidermis (16). In the absence of buffer, two phases of extension could be kinetically discriminated, a rapid initial phase and a slower phase with a peak after about 30 min (see Fig. 5). Superimposed on this spontaneous extension, an acid-induced extension response with a maximum after about 5 min could be resolved from the elongation-rate kinetics (Fig. 3a). In peeled segments, the acid-induced response was about twice as large as in nonpeeled (abraded) segments. Nevertheless, there was no shift in the pH-dependency to higher pH upon peeling (Fig. 3b). Thus, on a relative scale, the responses of nonpeeled (abraded) and peeled coleoptile

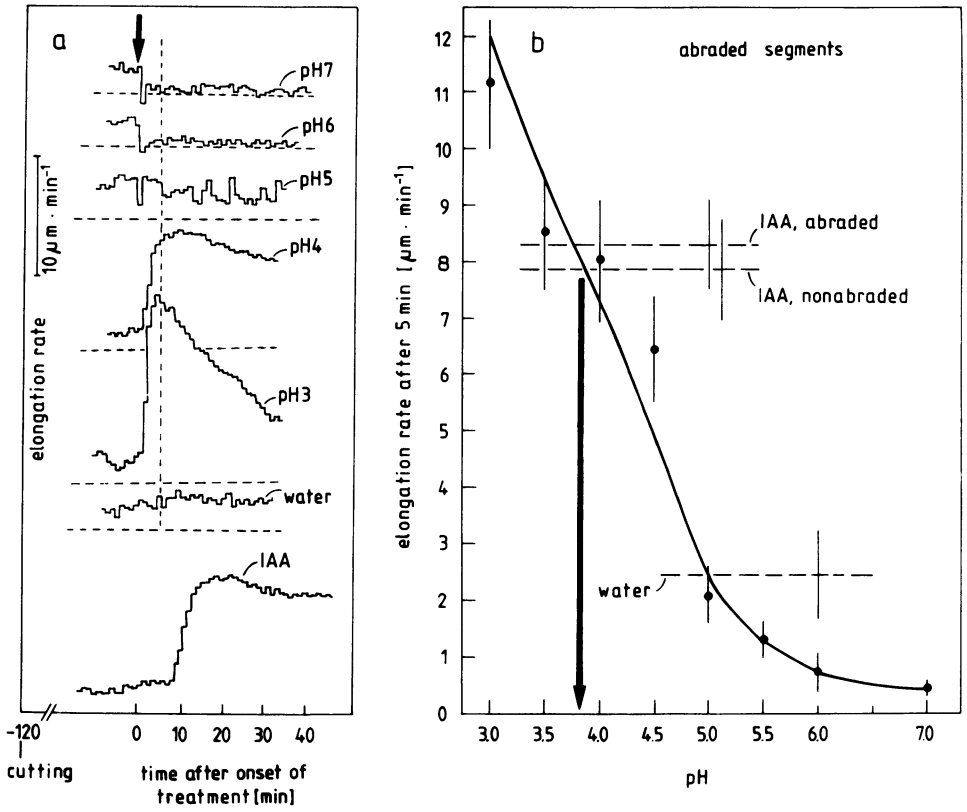


**Figure 2.** Effect of acidic buffers and IAA on elongation of abraded *Avena* coleoptile segments. a, After 2 h of preincubation in water and 10 min of osmotic adaptation in mannitol (20 mmol $L^{-1}$), the segments were transferred (arrow) to iso-osmotic Na-citrate buffers (citric acid/Na-citrate, 7 mmol $L^{-1}$ citrate) or IAA (10 µmol $L^{-1}$, supplemented with 20 mmol $L^{-1}$ mannitol). b, Maximum acid-induced elongation rates (reached after 5 min, see vertical broken line in (a) are plotted as a function of pH and compared with the maximum elongation rate obtained by IAA treatment (after 20 min) in abraded and nonabraded segments (dashed lines). Abrasion had no significant effect on the elongation rate of the water control (containing mannitol).

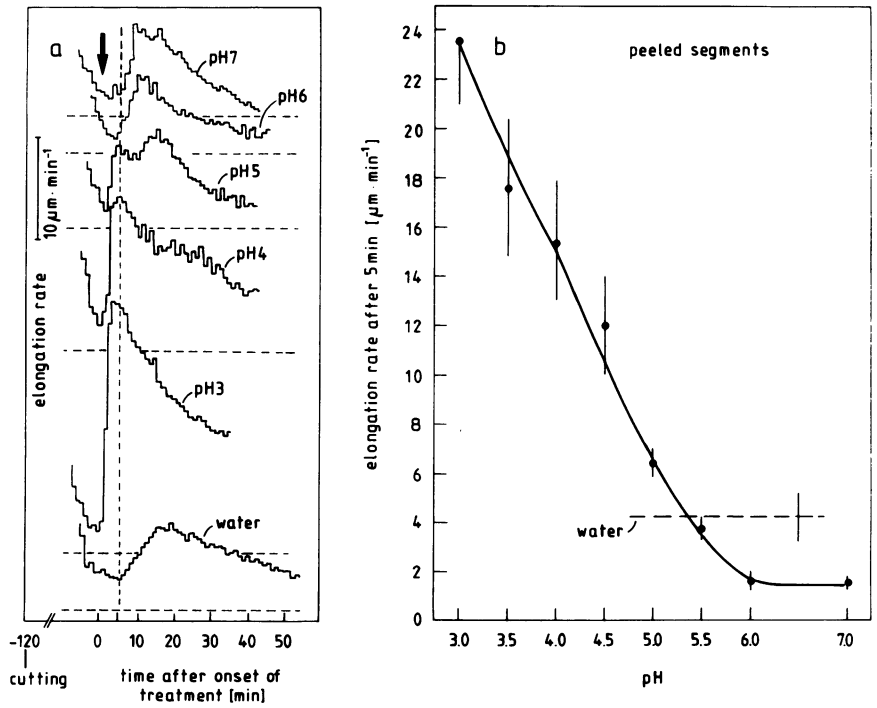


**Figure 3.** Effect of acidic buffers on elongation of peeled *Avena* coleoptile segments. a, After 2 h of preincubation in distilled water, 15-mm segments were peeled and reduced to 10 mm. After 10 min of osmotic adaptation in mannitol (20 mmol $L^{-1}$), the segments were transferred (arrow) to iso-osmotic Na-citrate buffers as in Figure 2. b, Maximum acid-induced elongation rates (reached after 5 min, see vertical broken line in (a) are plotted as a function of pH. The dashed line refers to the elongation rate of the water control (containing mannitol).

segments to external pH are virtually identical. This result agrees with data obtained with maize coleoptiles (16). It eliminates the possibility that the apparently much higher sensitivity of elongation to protons reported by Rayle (19) is due to the absence of the epidermis in his experiments.

Another experimental difference in the two sets of deviating data concerns the pretreatment of segments before eliciting elongation with acidic buffer. Rayle (19) preincubated peeled segments for 60 to 90 min in phosphate buffer (10 mmol $L^{-1}$) at pH 6.8 before switching to acidic buffer. In many subsequent investigations of this type it has also been customary to preincubate living or killed tissue in neutral buffer before

investigating acid-induced extension (*e.g.* 10, 13, 17, 21). In contrast, a preincubation in distilled water (+ osmoticum) was used both in the present investigation (Figs. 2 and 3) and in the related experiments with maize coleoptiles so as to remain as close to the natural apoplastic pH as possible (14, 16). The influence of a preincubation in neutral buffer on the pH-dependence of elongation in peeled segments is investigated in Figure 4. As shown in Figure 4a, a pretreatment with pH 6.8 buffer leads to an instantaneous burst of extension upon transfer to buffers of lower pH. Maximum rates occur about 2 min after transfer. This response was statistically significant at pH 6.0 and showed a first plateau at pH 4.5 to 5.5 and a second, slightly higher, plateau below pH 4 (Fig. 4b). In control segments preincubated in water, instead of pH 6.8 buffer, a subsequent transfer to acidic buffer elicited much smaller elongation rates with a threshold of responsiveness at about pH 4.5 and a linear increase toward lower pH (Fig 4b). These results demonstrate that the high sensitivity to pH 5 to 6 reported by Rayle (19) can be reproduced by pretreating the segments with neutral buffer. In fact, the pH-dependence of segment elongation depicted in Figure 1A of Rayle's paper (maximum rate at pH 5: 9 $\mu$m min$^{-1}$, ref. 19) is very similar to that in the region of the first plateau of the related pH-curve shown in Figure 4b (maximum rate at pH 5: 7 $\mu$m min$^{-1}$). (The second plateau at lower pH can only be resolved by measuring initial elongation rates [see Fig. 4a] and does therefore not show in Rayle's curve which was calculated from elongation rates measured 5 to 15 min after transfer to acidic buffer.) Thus, Figure 4 shows that the elongation response of *Avena* coleoptile segments to buffers in the pH range 5 to 6 is not a feature of the tissue itself but is experimentally produced by a preincubation at neutral pH. A similar effect of neutral buffer on the pH-dependence of acid growth has been shown in maize coleoptiles (14, 16).

How can a neutral buffer increase the sensitivity of coleoptile tissue to a subsequent treatment with weakly acidic pH? A possible explanation is hinted at in Figures 2b and 3b. The elongation of nonpeeled (abraded) and peeled segments is inhibited by pH 5.5 to 7.0 as compared to the water control. It appears conceivable, therefore, that the effect of pH 6.8 is in fact to suppress the spontaneous elongation which occurs in water, and that the removal of this suppression by transfer to a lower pH results in a transient release of extensibility stored in the presence of pH 6.8 buffer. This idea is further supported by the observation that, in pH 6.8-pretreated segments, water too can elicit an extension burst comparable to that elicited by buffers of pH 4.5 to 5.5 (see dashed line in Fig. 4b). This indicates that the first plateau of the pH-response curve for pH 6.8 preincubated segments is, in fact, not due to the action of external acidic buffer but to the removal of the neutral buffer from the tissue. Figure 5 shows this phenomenon more clearly. The removal of the mechanically constraining epidermis leads to a rapid transient extension by water uptake due to the release from tissue tension (16). Buffer of pH 6.8 inhibits this spontaneous response and transfer from pH 6.8 to water leads to an instantaneous release from inhibition. The inset in Figure 5 shows that this 'growth response' is not merely an elastic extension of the walls of peeled segments but contains an irreversible component also. Only this component is affected by the inhibitory action of pH 6.8 buffer.

It is evident from Figure 5 that the apparent induction of elongation by buffers of pH 4.5 to 6.0 in peeled coleoptile segments pretreated with pH 6.8 results from the physical

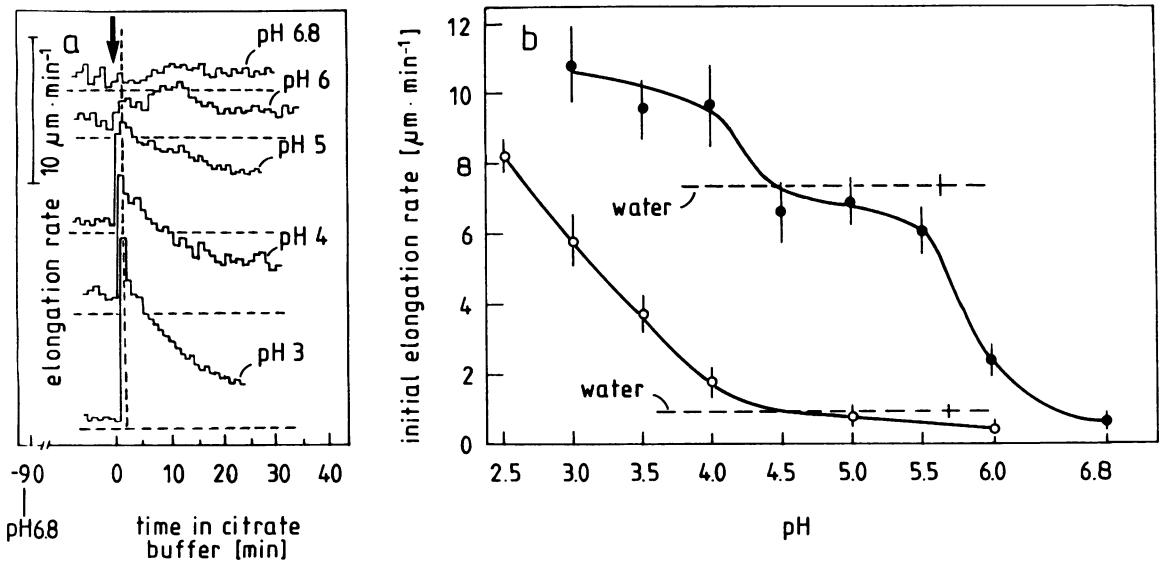


**Figure 4.** Effect of acidic buffers on elongation of peeled *Avena* coleoptile segments pretreated with neutral buffer (●) after Rayle (19) or with water (○). a, After 2 h of preincubation in distilled water, 15-mm segments were peeled, reduced to 10 mm, and incubated in K-phosphate buffer (10 mmol L$^{-1}$ phosphate [pH 6.8], ref. 19) for 90 min. Ten min after inserting a segment into the growth recorder, the phosphate buffer was replaced by Na-citrate buffers (7 mmol L$^{-1}$) of the indicated pH. b, Initial (maximum) elongation rates (reached after about 2 min, see vertical broken line in a) are plotted as a function of pH. The dashed lines refers to the elongation rate obtained upon transfer to water (containing 20 mmol L$^{-1}$ mannitol) instead of buffer. In a similar series of parallel experiments, the 90-min preincubation in phosphate buffer was replaced by a 90-min preincubation in water (containing mannitol, ○).

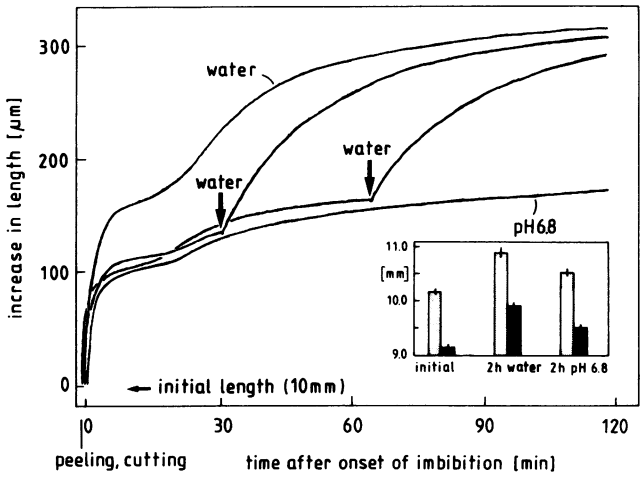


**Figure 5.** Effect of neutral buffer (pH 6.8) on the spontaneous extension of *Avena* coleoptile segments induced by peeling. After 2 h of preincubation in distilled water, 15-mm segments were peeled, reduced to 10 mm, and immediately attached to the growth recorder. Extension was started by immersing a segment either in water (including 20 mmol $L^{-1}$ mannitol) or K-phosphate buffer (10 mmol $L^{-1}$ phosphate [pH 6.8]). After 30 and 60 min the buffer was replaced by water (+ mannitol). The inset shows the total (light stipple) and irreversible (dark stipple, measured after freezing and thawing) length changes of peeled segments incubated for 2 h either in water (+ mannitol) or phosphate buffer. These measurements were carried out by eye at 20-fold magnifiction with a dissection microscope and are therefore not directly comparable to the growth-recorder data.

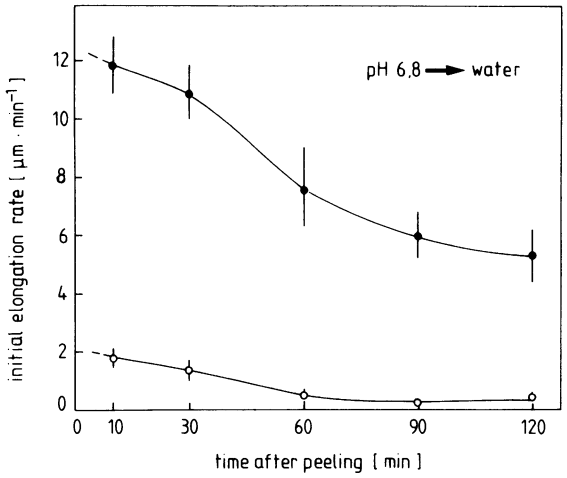


**Figure 6.** Effect of pretreatment time (10–120 min) in neutral buffer on subsequent extension of peeled *Avena* coleoptile segments in water. After 2 h of preincubation in distilled water, 15-mm segments were peeled, reduced to 10 mm, and incubated in K-phosphate buffer (10 mmol $L^{-1}$ phosphate [pH 6.8]). After the times indicated on the abscissa, the segments were transferred to water (containing 20 mmol $L^{-1}$ mannitol). Elongation rates were measured immediately before (○) and 2 min after (●) transfer to water (see Fig. 4a).

relaxation of the epidermal constraint which was temporarily stored under the influence of the pH 6.8 buffer. After this extension process has been allowed to take place in water, true acid-growth can be induced in peeled segments and this demonstrates the same low sensitivity to pH found in non-peeled segments (Fig. 4, open symbols).

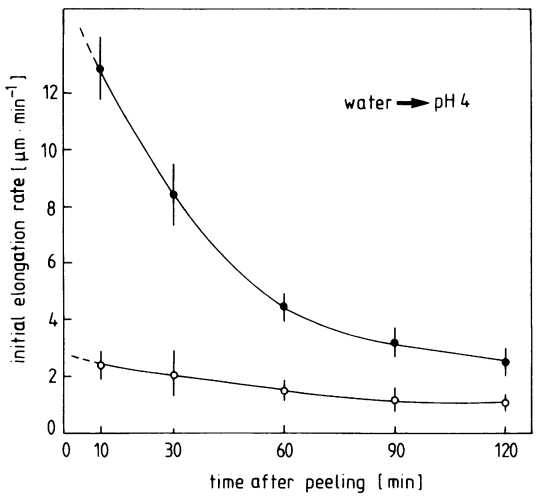


**Figure 7.** Effect of pretreatment time (10–120 min) in water (containing 20 mmol $L^{-1}$ mannitol) on subsequent extension of peeled *Avena* coleoptile segments in Na-citrate buffer (7 mmol $L^{-1}$ citrate, pH 4.0). Other experimental details as in Figure 6. Elongation rates were measured immediately before (○) and 2 min after (●) transfer to pH 4 (see Fig. 4a).

Figure 6 shows that the capacity for spontaneous extension upon transfer of peeled coleoptile segments to water only decreases slowly during 2 h of pretreatment with pH 6.8. In contrast, the capacity for true acid growth induced by pH 4 in water-pretreated segments decreases rapidly and has almost vanished after 2 h (Fig. 7). Obviously, some factor required for rapid growth is depleted during spontaneous extension in water but not in pH 6.8-buffer. These differences in the time courses of the two elongation responses provide additional evidence that the biochemical mechanisms underlying these processes are not the same.

## CONCLUSIONS

Segments from peeled *Avena* coleoptiles demonstrate a rapid, transient extension response which results from the elimination of mechanical stress exerted by the outer epidermal wall on the inner tissues of the organ (see Fig. 5). This phenomenon is an indication of the existence of longitudinal tissue tension (uneven tensions of outer epidermal wall and inner tissue walls) in the intact coleoptile, a general feature of growing axial organs (16, 22). In the presence of tissue tension, the longitudinal cell walls of the inner tissues are kept in a loosened state, permitting a limited amount of irreversible extension when the epidermal restraint is removed and the turgor drop (water potential decrease) leads to spontaneous water uptake and cell extension. This type of extension is not the same as the growth of the intact organ as the latter is controlled by the extensibility of the rigid, outer epidermal wall rather than the soft inner tissue walls (16). In order to initiate growth, the epidermal wall must first be *loosened* whereas the inner tissue walls merely need to be *extended* (16). It is thus questionable, whether peeled coleoptiles (*e.g.* ref. 10) are a suitable object for elucidating the mechanism of wall loosening responsible for the growth of the intact organ.

The yielding process and the accompanying extension response of inner tissues released from epidermal restraint de-

pends on the pH of the apoplastic solution and can therefore be experimentally promoted (inhibited) by lowering (raising) the pH in the range of 3 to 7. Whether a particular experimental pH causes promotion or inhibition depends on the pH of the wall solution prevailing before the pH change. It is important to note that it is the pH-dependence of extension rather than wall loosening proper which has been measured in these and all similar experiments with living or frozen-thawed material (*e.g.* ref. 10). The distinction between wall loosening and extension as separate processes in the course of cell-wall elongation was recently pointed out by Ray (18).

In the nongrowing, IAA-depleted *Avena* coleoptile, a physical extension response takes place spontaneously upon removal of the epidermis and incubation in water, *i.e.* in the absence of a major change in apoplastic pH which may be close to 5.0 (see Figs. 2 and 3 and refs. 2 and 3). Therefore, an experimental increase in apoplastic pH above 5.0, (*e.g.* to pH 6.8) inhibits the spontaneous extension response. The inhibition can be reversed instantaneously by washing the inhibitory solution out of the apoplast with water. This re-establishes the original slightly acidic pH in the cell wall, by, *e.g.* retention of respiratory $CO_2$. It is obvious that washing with a medium having a slightly acidic pH will have the same effect (see Fig. 4). It is this release of extension previously stored at neutral pH which has been mistakingly interpreted as acid-mediated wall loosening in terms of the acid-growth theory (6, 9, 19, 20). If intact (abraded) *Avena* coleoptile segments (which respond strongly to IAA after 12 min, see Fig. 1) are used for measuring acid-mediated extension, the sensitivity to acid is much lower. There is no growth-promoting effect above pH 5.0 and the pH required for mimicking the IAA-mediated growth rate is close to 3.5, *i.e.* beyond the physiological range (see Fig. 2). The low sensitivity to external acid solutions cannot be explained by a low permeability of the epidermal surface to protons since peeled segments demonstrate a pH-response curve very similar to that of intact (abraded) ones (see Fig. 3). Thus, the pH-dependence of acid growth in *Avena* coleoptiles is the same as that previously reported for maize coleoptiles (14, 16) and there is no reason to doubt that the basic arguments raised against the acid-growth theory of auxin action in maize (14, 15) also apply for *Avena*. Furthermore, all attempts to demonstrate a pH-optimum of growth in the range of auxin-mediated wall acidification in dicot seedling stem tissues have either failed (1, 2, 25) or led to inconclusive results (17, 21). It must therefore be concluded that the acid-growth theory of auxin action has so far not been confirmed experimentally in any plant tissue.

## ACKNOWLEDGMENT

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